A NEW GENERATION OF ATMOSPHERIC TRACERS — THE BIOLOGICAL CONNECTION

Final Report

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from

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A prototype atmospheric tracer system (ABS-1) has been developed that utilizes insect pheromones as the tracer and a live insect as the atmospheric biosensor (ABS-1). The laboratory experiments indicate that not only the presence of the phermone tracer can be detected, but also an estimate can be made of the tracer concentration. Thus, atmospheric transport and diffusion estimates should now be possible with the ABS-1.

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I. INTRODUCTION

The use of atmospheric tracers can provide information on air flow features that cannot be directly observed by meteorological instrumentation systems. Even in the special situations where remote sensing systems can operate, sufficient aerosol-hydrometer backscatter is required before relatively short-range volume scans of the velocity field are possible. However, all measurement systems (standard or research) are deficient in being able to directly measure the Lagrangian features of the atmosphere. Tracers, on the other hand, can be designed and used to specifically document many of the actual flow characteristics (transport/diffusion) of the atmosphere. Consequently, atmospheric air motions or circulations do not have to be deduced from point measurements separated in time and/or space or from analytical or numerical models based on point measurement parameterizations. As a result, tracer techniques can be extremely useful in deducing short or long-range transport and diffusion of atmospheric constituents within or near boundary layers, convective elements (dry or moist), complex terrain, inversions, etc.

While the use of tracers provides in concept a sound scientific approach for documentation of atmospheric transport and diffusion charactgeristics, the anticipated results are usually not attainable because of several basic or inherent deficiencies which can act to 'cloud' the experiment(s). We believe that the use of tracer techniques will truly enter the 20th Century when there is a significant improvement in the following three, basic tracer requirements:

(1) Continuous, real-time sensing capability,

(2) High sensitivity of sensors,

(3) Low atmosopheric background of tracer material.

Several other tracer requirements must also be met; i.e. tracers should be:

(4) Nontoxic and produce no adverse environmental effects,

(5) Low cost,

(6) Capable of following air motions without significant removal or reactions with other atmospheric constituents,

(7) Easy to handle and release in the atmosphere.

The first three tracer requirements have the highest priority because they involve both the probability and accuracy of detection [(2) and (3)] in a manner that provides both space and time coordinates of the associated atmospheric motions [(1)]. State-of-the-art samplers today have either sacrificed [(1)] in favor of [(2) and (3)] (i.e. bulk samplers) or vice versa [(i.e. sacrificed (2) and (3) in favor of (1)]. In order to eliminate this dichotomy of detection performance, we have been experimenting with tracer systems that will equally satisfy all three high-priority, basic tracer requirements. One of these systems employed the airborne release of submicron cesium particles with subsequent real-time, continuous plume tracking by fast response ionization detectors (Sinclair and Finnegan, 1981). Although all the alkali metals (Li, Na, K, Rb, Cs) can easily be detected by the mobile SIMP-1 (Surface Ionization Monitor for Particulates), cesium was selected because of its low ionization potential (provides high sensitivity and isolation) and its low atmospheric aerosol background concentration ($\sim 10^{-9}$ gm m^{-3}). It is a low cost tracer system that can release the tracer material from a ground or airborne platform (aircraft, balloon, kite, rocket, etc.) at a controlled rate from a few seconds to several hours. This system was successfully tested and used to validate the fast-response plume dispersion models employed by the Defense Nuclear agencies. However, in our efforts to improve the sensitivity and short-range transport features of the cesium system, we have found that an entirely different approach to the development of a high-performance, low cost tracer system is not only feasible but highly probable. This new concept involves the use of living biological sensors which have the potential of greater sensitivity than the best detectors available, such as those used with sulfur hexafloride (SF_6), methane-21 ($^{13}CD_4$), or the perfluorocarbons (Cowen, *et al.*, 1975; Johnson, 1983; Baxter, 1982). The extensive research by entomologists over the past

35 years provides a firm foundation for the development of a high-performance, low cost atmospheric tracer system using live insect receptors and pheromone compounds.

II. PREVIOUS LABORATORY RESEARCH

A. Early History

It has been known for many years that animals use their olfactory capabilities as a powerful chemical communication link between their own species (sexual attraction, trail following, recruitment, and defense) and outside their species with their plant and animal hosts. During the 18th Century several very important observations and tests of male moth communication capabilities were performed:

1. Several naturalists such as Réaumer, Lesser, and Rösel von Rosenhof described the male moths impressive capability to locate their female partners over distances of several hundred meters (Forel, 1910).

2. Fabre (1914) using marked male moths of the Chinese silkworm moth (*Actios selene*) showed that they could locate females in a gauze cage at distances from 4.1 to 11 km. Using Gypsy moth males, Collins and Potts (1932) also claimed that the males could locate a female moth from a distance of 3.8 km.

While these experimental results in the field of chemical ecology may have had an aura of the anecdotal, the exotic, and the impractical, they provide the basis for our present belief that: if the concentration of the sex attractant[1] from a single female moth can lure a male moth from a distance of 1–10 km, then a suitable increase in the attractant could result in an increase in the detection limits to possibly 1000 km or more. The feasibility of this detection capability has great promise when one notes that the quantities of chemical messengers from the female moth range from only a few hundred molecules (Kaissling and Priesner, 1970) to a few micrograms. Since the pheromone compound is a hydrocarbon

[1]The term "pheromones" was coined by Karlson and Lüscher (1959) to designate the chemical messengers used by insects in intraspecific communication.

structure ($C_{16}H_{30}O$, $C_{19}H_{35}O$, etc.), it can be easily dissolved in say pentane, butane, etc. and sprayed into the atmosphere in quantities detectable at much larger distances than the normal insect range. The carrier evaporates quickly, leaving the pheromone in a gaseous state.

This early discovery of pheromone communication between insects at long distances (1–10 km) was followed by many years of uncoordinated and disruptive (World War II) research concerning the identification of site(s) of both preception and production of the sex pheromones and devising methods to determine their species specificity and their chemical nature. This situation radically changed with the discovery, isolation, and chemical identification of the silkmoth pheromone by Butenandt, *et al.*, 1959. The compound was identified and synthesized as (E)10-,(Z)12-Hexadekadiene-1-01 and given the name Bombykol (Butenandt, *et al.*, 1959, 1962). Today, after this pioneering contribution over 800 substances have been described and chemically synthesized. In addition, techniques for measuring the insect response to its particular pheromone compound have improved markedly from the early development of the electroantennogram (Schneider, 1957) to present day techniques: (1) combined gas chromatography and electroantennogram detectors (GC-EAD) (Struble and Arn, 1984); (2) coupled gas chromatography—single cell recording (GC-SCR) (Wadhams, 1984); (3) coupled gas-liquid chromatography—mass spectrometry (GLC-MS) (Gohlke, 1959; Ryhage, 1964); (4) tandem GLC-EAD (Moorhouse, *et al.*, 1969; Arn, *et al.*, 1975; Wadhams, 1984); and (5) the tandem GLC—behavior bioassays (t-GLC-BB) (Hummel, 1984).

These techniques have provided crucial information for basic insect pheromone research. In addition, the urgent need of insect pest control measures which are more specific and less harmful than the general use of insecticides acted as a catalyst for the experimentation and use of pheromones for insect control. As a result of this combined basic and applied research effort over the 25 years following Butenandt, *et al.* (1959) isolation of Bombykol, we have developed the prototype of a new generation of atmospheric tracer systems using pheromone compounds and insect receptors. The advantages of this biological connection

takes us into a completely new realm of tracer technology with significant improvement in all tracer system requirements [(1)–(7)].

B. Insect System

In order to understand the prototype atmospheric tracer system, an explanation of the pheromone communication system as it exists in the natural insect world is presented for the female sex attraction system of the *Bombyx mori* (silkworm). the calling female evaporates a specific pheromone compound or mixture from a modified and enlarged intersegmented membrane near the tail of the insect. This minute quantity of pheromone molecules is carried by atmospheric motions to the receiving male moth located some distance downstream. It is important to note that the female moth may contain only 2×10^{-8} gm of pheromone compound(s) at any one time of which only 3×10^{-12} gm are required to elicit significant response from the male. As a result, the detection system of the male moth operates on the molecular scale and not on the customary macroscale of man-made, atmospheric sensors and detectors. The following description of the male moth detection system follows directly from Steinbrecht and Schneider, 1980.

The male moth intercepts the pheromone molecules via several antenna approximately 6 mm in length (Figure 1). Each antenna has a large outline area due to side branches of the flagella which carry 17,000 long sensilla trichodea (ST) in a regular and dense array (Figure 1, f–h). These structures filter out a large portion of pheromone molecules from the passing airstream (Kaissling, 1971). Moreover, the dimensions of the sensory hairs (length 100 μm, mean diameter 2 μm) are such that adsorption of odor molecules by convective diffusion preferentially occurs on the hair surface pores (P). The thousands of 100 angstrom pore tubules (PT) in the hair wall are considered the routes along which the stimulus molecules diffuse to reach the receptor dendrites (D) in the hair lumen (Figure 1i). Some of the pore tubules directly end in contact with the dendritic membrane (Figure 1j) (Steinbrecht, 1973).

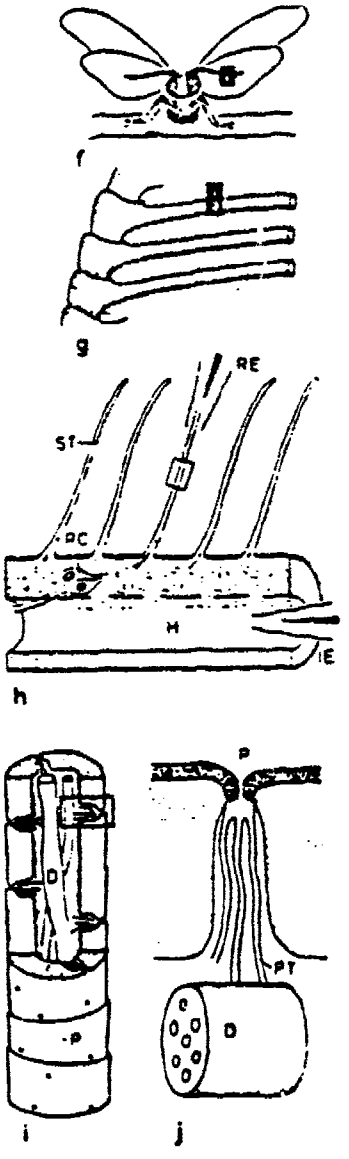


Figure 1: Pheromone communication in *Bombyx mori*: f–j, the male receiver (frames indicate subsequent enlargements); see text for explanation.

Each of the sensilla trichodea (ST) on the male antenna comprises two receptor cells (RC). One is stimulated maximally by bombykol and much less by its stereoisomers; the key compound of the other is bombykal (Kaissling, 1977; Kaissling, *et al.*, 1978). Thus, the specificity of the reaction cannot be explained by different specificities of the stimulus conduction (adsorption and diffusion of odor molecules), but is given by the receptor molecules in the receptor membrane, which interact with the stimulus molecules, possibly in similar ways as in other processes of molecular recognition (for references see Kaissling and Thorson, 1980). In the electrophysiological experiment (Figure 1h) where the recording electrode (RE) is slipped over a hair with cut tip and the indifferent electrode (IE) is inserted in the hemolymph (H), the signal-receptor interaction is indicated by a depolarization of the receptor membrane. The amplitude of this receptor potential increases with stimulus strength and correlates with the frequency of the nerve impulses propagated to the brain of the male moth. in *Bombyx* and presumably in other moths as well, a single pheromone molecule may elicit a nerve impulse, but several hundred receptor cells have to be activated to overcome the noise of spontaneously firing receptor cells and trigger the behavioral reaction (Kaissling and Priesner, 1970). the sensitivity and specificity of the male's antenna to its pheromone component make it a powerful tool in assaying for pheromone components and in predicting the structure of the pheromone components.

As a result of this very selective and sensitive detection system, the male silkmoth when stimulated with only a few hundred bombykal molecules begins an upwind flight in the odor plume toward a luring female which may be as far as 11 km away. We have used this astounding sensitivity of pheromone communication in moths (and also other insects) to provide a foundation for development of a new thrust of basic research in atmospheric tracer systems.

C. Laboratory Detection Techniques

Because of the great sensitivity of the insect antenna to pheromone molecules, we have made direct use of the male antenna system in the detection of pheromone tracers released

in the atmosphere for the the study of atmospheric motions. The antenna system of many insects are easily accessible and have been studied in great detail (Steinbrecht and Schneider, 1980; Kaissling and Thorson, 1980; Kaissling, 1971, 1979). Schneider (1957) showed that slow olfactory receptor potentials could be recorded and that the amplitude of the response was positively correlated with the chemical stimulus concentration. The electroantennogram (EAG) technique, pioneered by Schneider, provides a real-time, continuous capability for detecting and recording the insect olfactory receptor potentials that are stimulated by the pheromone compounds.

A simple but typical EAG experimental system is illustrated in Figure 2. In general, the EAG set-up consists of electrodes, a high input impedence (10^{12} ohm) amplifier, and a recording device. Tungsten or Ag/AgCl recording electrodes are connected to one end of the insect antenna—the other end is grounded in a saline solution to complete the circuit. The recording electrode is shielded with aluminum foil to reduce noise interference. An odor stimulus causes a negative deflection of the receptor potential, which rises relatively fast and declines more slowly after the end of the stimulus as shown by the oscilloscope trace. An EAG experiment can use the entire insect, the head plus antennae, or a single, plucked-off antenna. We have shown that if the antenna is not detached from the insect, recordings can be made for several weeks.

D. Signal Amplification

During our early research on the pheromone tracer system, we had envisioned that several male antennae could be connected in series to increase the electrical output. This could provide an internal amplification factor as well as provide an antenna array that would collectively sweep-out, from the airstream, a more representative pheromone sample. Much to our gratification this "biological amplification" has already been demonstrated by Moore (1981) in screening crude female extracts for EAG activity response. His results agree with and extend the summation theory which equates the magnitude of the EAG response from a given stimulus to the number of receptors reacting (Payne, 1975). Moore's EAG results

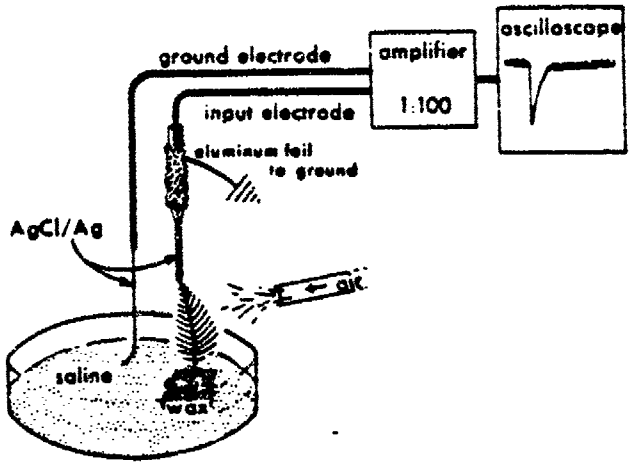


Figure 2: EAG laboratory test configuration of a single antenna fixed in wax. The antennal base makes contact with ground through a saline solution, and the distal antenna tip makes contact with saline solution in the input electrode tip. Test chemicals are puffed into an airstream that continuously passes over the antenna (from Roelofs, 1978, 1980, 1984).

are shown in Figure 3 for the male *Spodoptera littoralis*. Two female sex pheromones (I and II)[2] were used separately and in combination to determine the functional relationship of the EAG response amplitudes to the component blending and source doses. The purpose of this research was based on the results of Priesner (1979a) who found that within the receptor system of the male *S. littoralis* there were two specialist cells reacting to components I and II. In addition, the field work of Kehat, *et al.* (1976) showed that a 100:1 blend of I:II could significantly increase male catches.

From the results presented in Figure 3 it is apparent that there is a significant and rapid rise in EAG magnitude with the number of antennae connected in series. In addition, Moore found that the four antennae in series gave significantly greater depolarizations with the binary mixture (I and II) than with the major component (I) alone. Also, these results and others showed that through the use of multiple antennae connected in series and various source doses (0.02 to 20.0 μg), one could more easily detect the presence of two specialist

[2]Primary pheromone component I: cis-9, trans-11-tetra-adecadienylacetate (A,E-9, 11-14:OAc) (Nesbit, *et al.*, 1973; Tamaki and Yushima, 1974); secondary component II: cis-9, trans-12-tetra-decadienylacetate (A,E-9, 12-14:OAc) (Tamaki and Yushima, 1974).

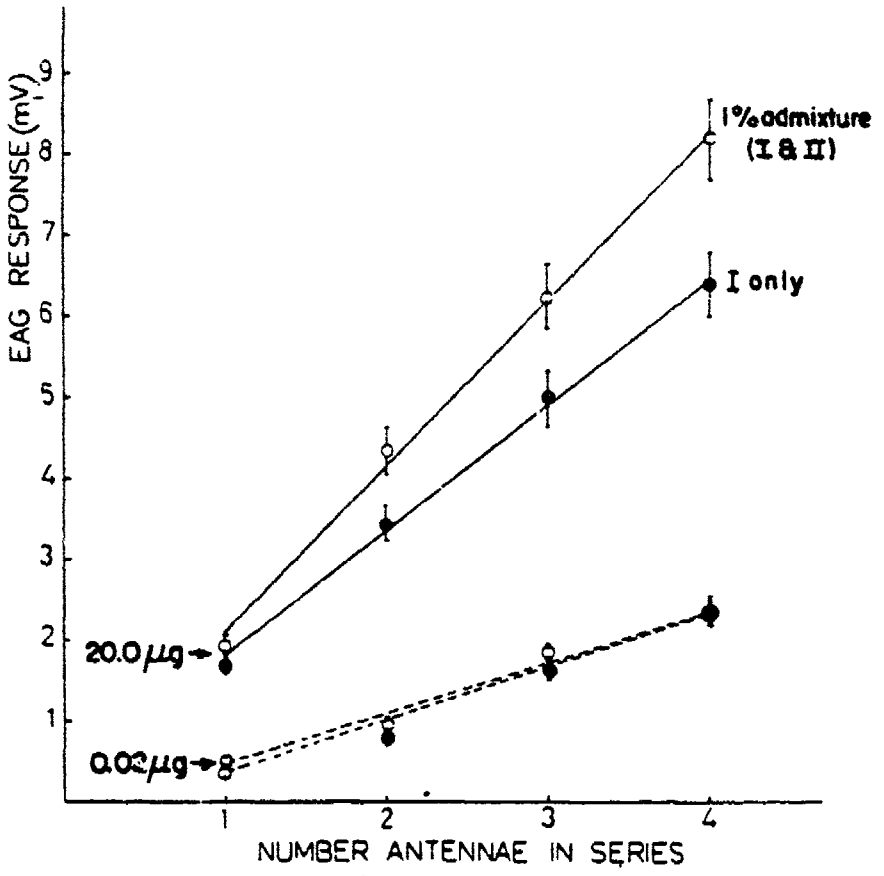


Figure 3: Signal Amplification by Antenna Arrays. EAG responses of male *Spodoptera littoralis* antennal preparations to 0.02 μg (- - -) and 20.0 μg (—) source concentrations of Z,E-9,11–14:OAc alone (•) and with 1% Z,E-9,12–14:OAc admixture (o). Each dot is the mean of 16 readings. Vertical bars represent standard errors. (From Moore 1981).

receptor cells found by Priesner (1979b).

These results are very significant with respect to the proposed pheromone tracer system in that they offer an internal mechanism to increase the combined male receptor response to external stimulation and at the same time improve the signal-to-noise ratio. In addition, Moore's results show that the largest response ratios (I vs II) were obtained with the 0.2 and 2.0 μg doses—this can be useful in the preliminary design of the atmospheric doses or concentrations required for optimum receptor response.

E. Concentration Measurements

In atmospheric tracer studies of transport and diffusion phenomena, it is important, in addition to detection of the tracer to be able to measure the tracer concentration in order to assess the dispersive features of the flow and/or the transformation or removal of the pollutant of interest. Recently, Mayer, *et al.* (1984) in a quantitative study of the EAG of *Trichoplusia ni* (Hübner) response to a sex pheromone have provided new quantitative results that show:

1. the EAG is linearly proportional to the number of antennal sensilla stimulated and

2. the EAG is related to pheromone concentration by a power law function.

These results are extremely important to the proposed atmospheric tracer system in that one does not have to deal with pheromone dose (μg) sources for antenna calibration and laboratory tests. That is, it is now possible to determine the pheromone concentration ($\mu mole/cm^3$) in the stimulus air environment (Mayer, *et al.* submitted to *J. Chem. Ecol.*). Using this technique Mayer has shown that the stimulus-receptor response curve for the major component of the *T. ni* pheromone, (Z)-7-dodecen-1-oiacetate (Z7:12AC) is a statistically significant fit to a power function as shown in Figure 4.

Mayer's results not only suggest important differences between the power function (Mankin and Mayer, 1983; Stevens, 1975) and the previously accepted log-linear relationship for receptor generator potentials and pheromone stimuli, but also show the effect of

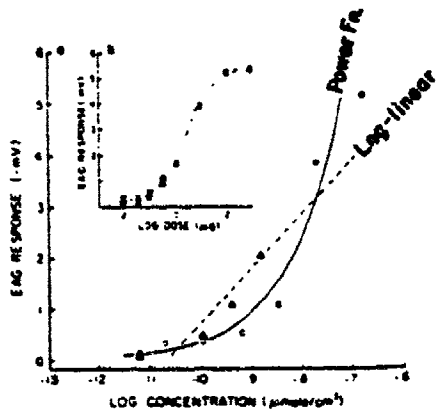


Figure 4: Pheromone Concentration Measurements from EAG Response. (a) Mean antennal polarizations of *T. ni* males at different concentrations of Z7:12AC. (b) Mean antennal polarizations at different dispenser doses. Note that the release rates of the 10^2 and 10^3 μg doses in (b) were equal so that they merge in (a). Symbols: open circle, mean responses of 19 males obtained at dispenser flow of 50 ml/min and carrier flow of 140 ml/min; filled circles and triangles, mean responses of 15 males obtained at dispenser flow of 200 ml/min and carrier flow of 1200 ml/min (*the different symbols indicate that different calibration equations were applied in converting a dose level of Figure 4b into an equivalent concentration level of Figure 4a*); solid line, power function of best fit; dashed line, log-linear function of best fit.

saturation levels on EAG response—this again will be useful in developing tracer sources release rates for various atmospheric conditions.

III. ATMOSPHERIC BIOSENSOR (ABS–1) DEVELOPMENT

A. Design Requirements

Although much of our preliminary research followed the standard entomologist laboratory approach, it was quickly recognized that an atmospheric device, deployed in the field for extended periods must have quite different design requirements than that associated with the usual laboratory device. For example:

1. The EAG connections must be of rugged design to withstand considerable vibration from transport and field deployment.

2. The antenna must have a life-span of several weeks to be useful for field measurements.

3. The entire biosensor (including amplifiers and slip rings) must be small and compact to fit within a small wind vane system or airborne probe so as to sample the prevailing wind flow for the pheromone tracer.

4. The pheromone compound released for transport and/or diffusion tests must be sufficiently inexpensive to economically operate over a long period of time.

5. The biosensor should be designed and calibrated to detect and record the presence and concentration of the released pheromone compound.

We have essentially accomplished all of the above design requirements that were initially specified in the proposal. These results of this investigation are discussed below:

B. Biosensor (ABS-1) Prototype Design

This initial laboratory research indicated that current electrical attachment techniques (Figure 2) used by entomologists to monitor the EAG voltage variations would not be adaptable to nonlaboratory applications. In addition, the insects normally used in these tests are very small (gypsy moths, western corn root worm, etc.) and consequently require very delicate antenna attachment techniques and apparatus. Consequently, we have developed a prototype biosensor model which incorporates noninvasive, hard-point attachment of the electronic circuitry to the insect antenna. We have selected the American cockroach for these initial tests because of its large antenna/body system. To our knowledge this has not been accomplished by previous investigators. Also, at this point in the research it was evident that the complete insect must be used in order to maximize the insect's life-span. That is, the common entomological practice of removing the antenna from the insect was not acceptable since the blood supply to the antenna was stopped and hence the voltage output from the antenna was reduced to a duration of only a few minutes. Consequently, the environmental control and feeding of the insect became an important prototype design factor.

The prototype biosensor is housed in a 2-inch diameter, 5-inch long tube structure which is designed to not only hold the insect but also provide space for the electronic circuitry, i.e.,

voltage amplifier, antenna 'hard' connections, air flow ducting, and feeder system (Figure 5). The insect (cockroach) is held in a fixed position that will allow eating and drinking to prolong its life-span. Initial tests show that the cockroach can survive for 22 days without food or water and 56 days when only water is supplied. A technique was developed to reduce the risk of damaging the antenna while handling the insect prior to recording. This was accomplished by constructing a jacket platform or 'body bag' to hold the roach for electrophysiological recording (Figure 6). Two pieces of stiff white cards were cut, one measuring 2.5 x 5 cm, and the other measuring 2.5 x 10 cm. The small piece was placed on top of the large one, the ends were aligned flush with one another, and the two pieces were taped together along the long sides of the short piece. This created a tube into which the roach could be introduced head first, with the long card piece providing support for the antenna. Before the roach was introduced, two pieces of tape were used at the upper end of the tube to create a hole just large enough for the head of the roach to slip through, yet small enough to block the body from slipping through. After the roach was introduced into the tube, the end of the tube was cut flush with the end of the roach body, and the tube was taped at the lower end to prevent the roach from slipping out backwards. This 'body bag' procedure made it possible to limit the movements of the roach so that it could not tear the electrodes from the antennae. This technique reduces the insect installation or hook-up time by a factor of at least five, i.e. to approximately 3 minutes. Below the insect's mouth (not shown in Figure 6) is located a small insect 'feeder' which holds water and ground dog food in a sponge environment for anti-slosh control.

This cylindrical mounting unit for the cockroach is housed in a small, light-weight wind vane in order to orientate the air inlet into the wind (Figure 5). The air flow is directed through the duct system over the antenna and exits out the top of the wind vane body. The design then provides a means to determine the mass flow of the air plus the pheromone tracer, past the antennae.

Probably the most important feature of the ABS-1 is the design of the EAG 'hard' connections (Figures 6 and 7). The design of the noninvasive, hard-point attachment of

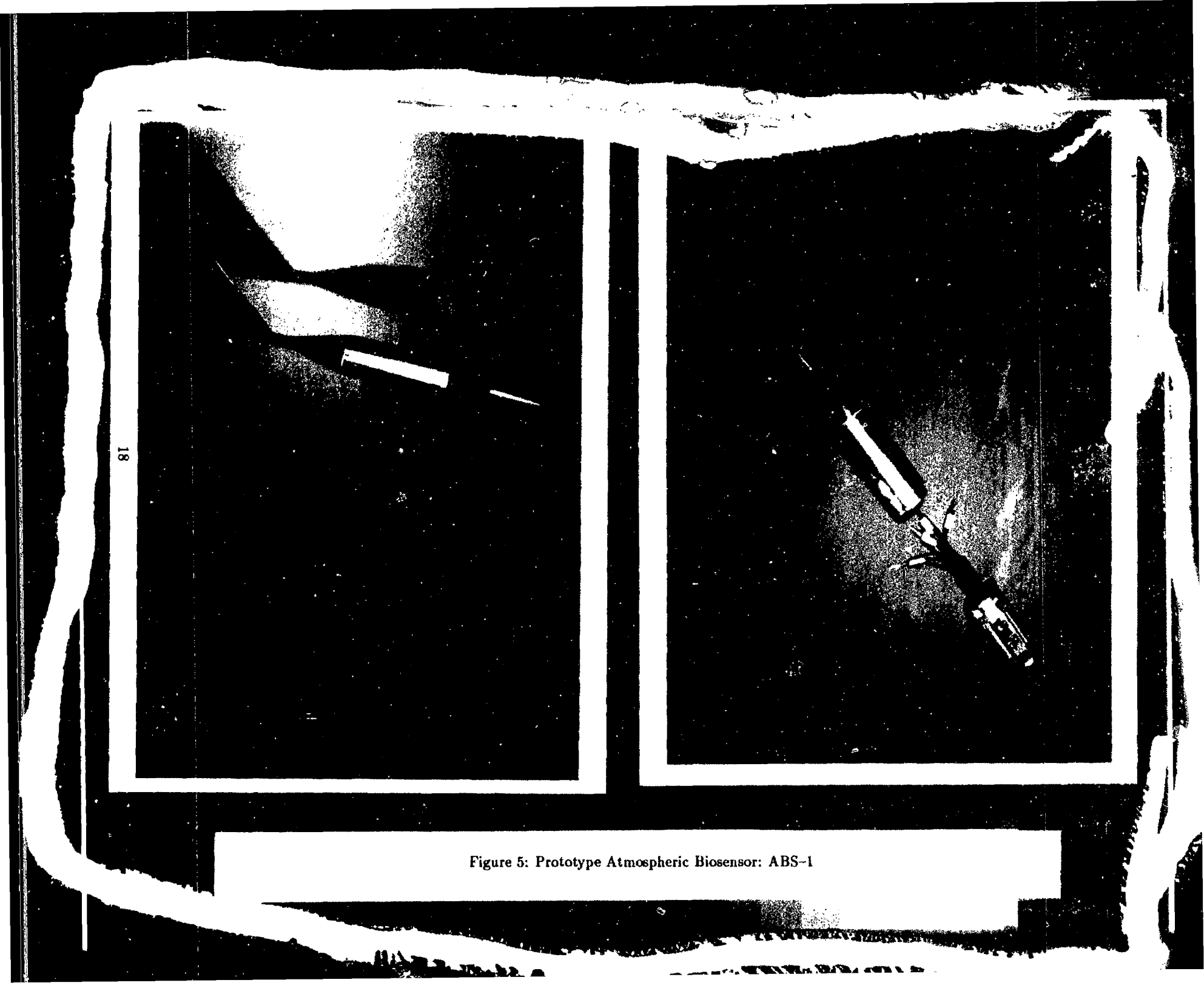
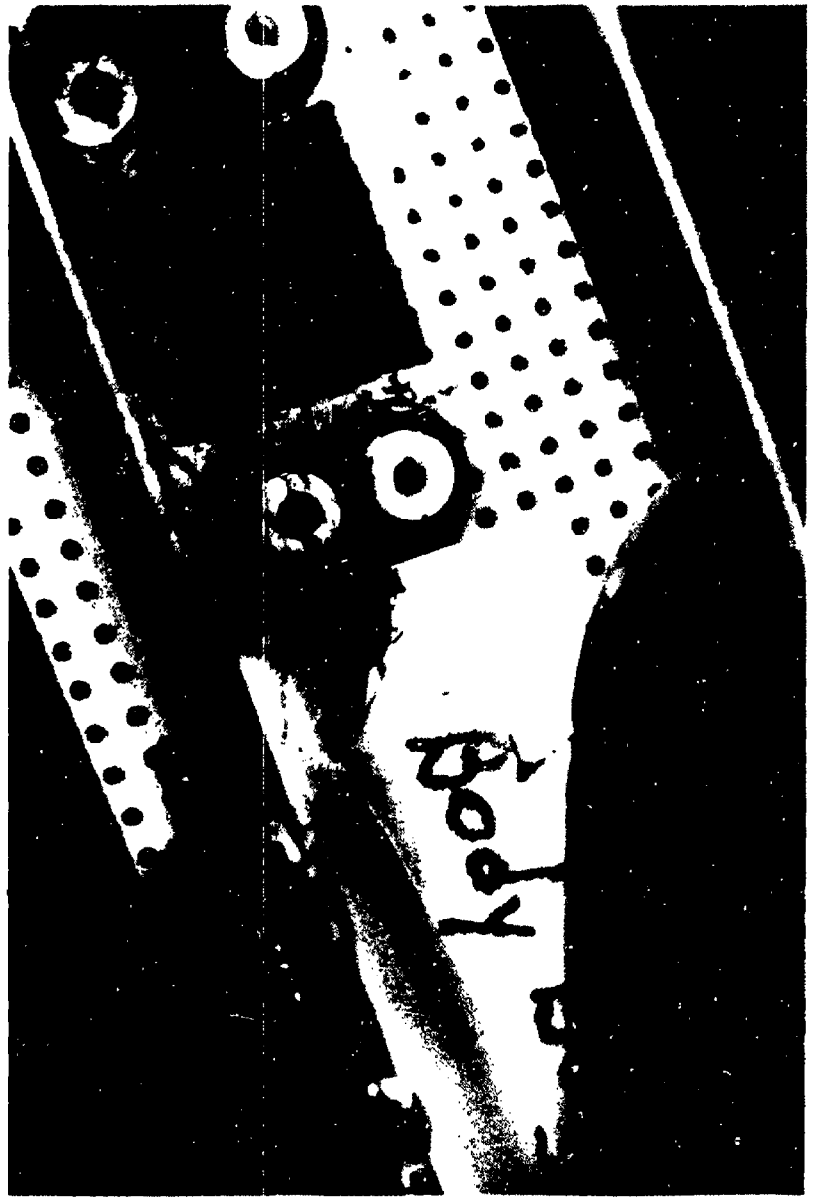
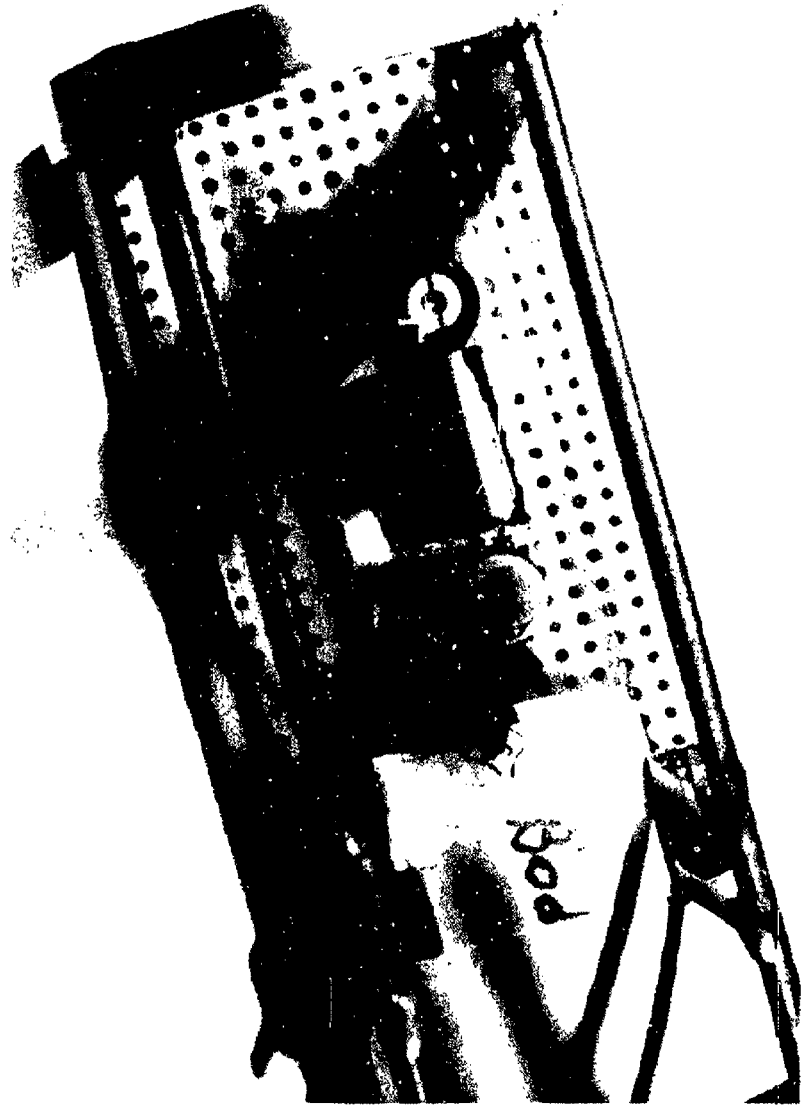


Figure 5: Prototype Atmospheric Biosensor: ABS–1

Figure 6: Insect 'Body Bag' and 'hard connections.





the electronic ciruitry to the insect antenna utilizes two (for each antenna) 1/8″ (I.D.) plastic tubes filled with EKG conducting gel (brand name SCAN). We previously used 0.1 M KCl, according to Roelofs (1984). Because this solution tends to dry out over several days, we wished to try saline media that would resist dessication. An excellent alternative has turned out to be one of the gels designed for medical use (SCAN Ultrasound Gel, Parker Laboratories, Inc., Orange, New Jersey 07050). This gel allows excellent EAG recordings, yet does not dry out appreciably if the electrodes are sealed to the antenna as described below. The SCAN filled tubes (Figure 7) have small slits on one end to allow the antenna to be inserted and held in place by a '0' ring plug (Figure 6). Inside the gel filled tubes a platinum wire coil is located just below the antenna. It is connected by shielded wire to the high gain (1000x) differential amplifiers in thc solid rear cylinder of the insect mounting structure (Figures 5 and 7). The gel acts as the conducting medium between the antenna and the platinum wire coil. The '0' ring stoppers seal the connection to prevent gel evaporation and to pin down the antenna with sufficient pressure to minimize electrical noise and mechanical vibration effects. This design has better performance than initial designs using saline solution as the conducting medium between the antenna and the coil pick-up. It was found that the saline solution not only evaporated more easily but it also poisoned the antenna and the insect in a matter of a few hours to a day. On the other hand, the SCAN gel did not cause problems over a period of 56 days where only water was supplied to the cockroach.

A second noninvasive approach was also developed in the laboratory to eliminate the use of sharply pointed glass capillary electrodes to pierce the outer cuticle of the antenna. This latter procedure has two drawbacks. First, piercing the antennae shortens the lifetime of the electrophysiological preparation. Second, the saline medium tends to dry out around the periphery of the electrode tip. We developed a different recording technique that avoids these problems. For the reference electrode, a short annulus of glass tubing is slipped over the end of the intact antenna and is slipped all the way to the base of the antenna. The annulus is filled with ultrasound gel (as described above). The tip of a 2 cm piece of platinum

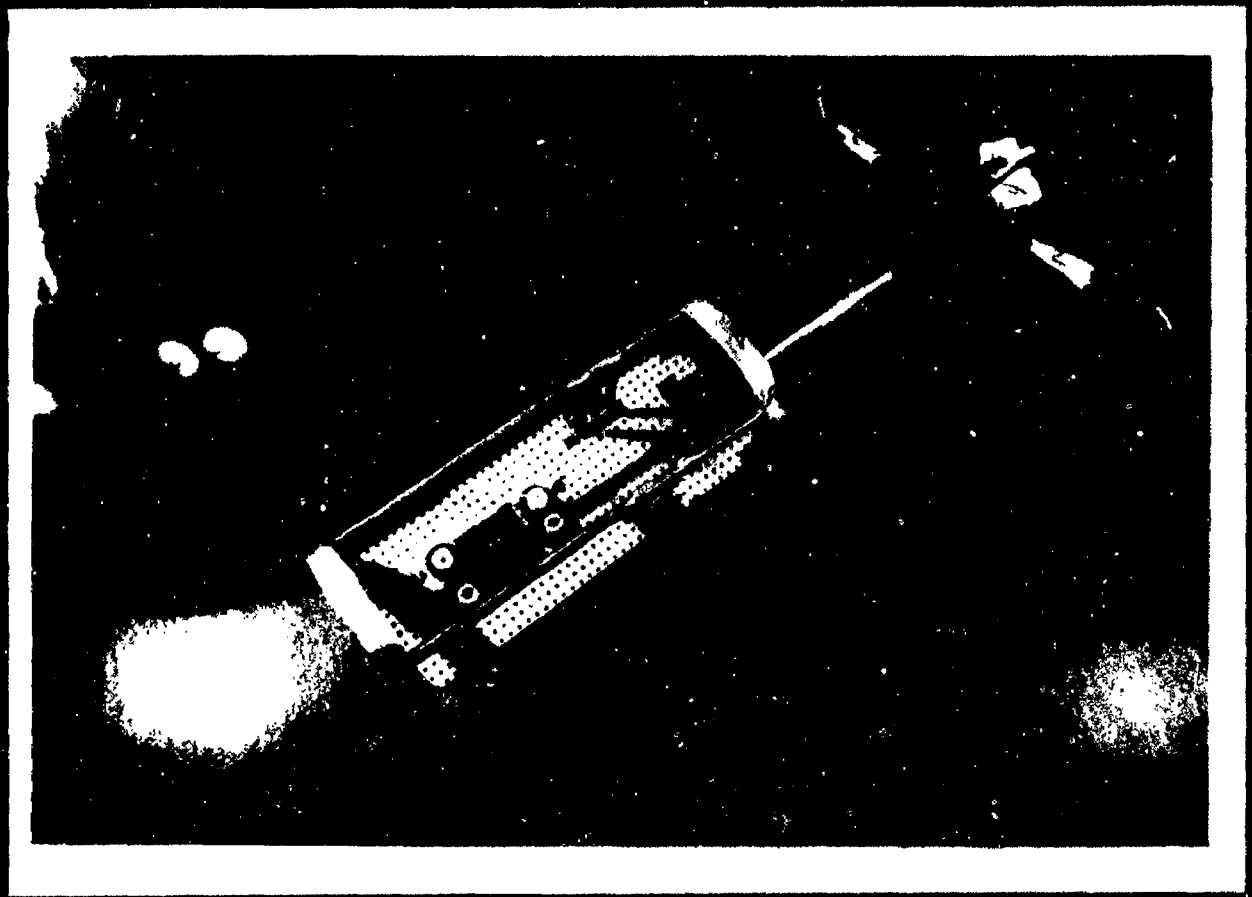


Figure 7: SCAN Ultrasonic Gel Conductor Tubes.

wire is inserted into the gel, and the ends of the annulus are then sealed with bayberry wax (melting point 42–48°C). Bayberry wax is so low-melting that is provides an adequate seal without causing damage to the antenna. For the recording electrode, a similar annulus is slipped over the very tip of the antenna dn sealed in place. For this electrode, one end of the annulus can actually be melted around the platinum electrode as an alternative to sealing in place with bayberry wax. Both of these noninvasive antenna hook-up procedures can be used in field designs of tracer systems.

C. Insect Pheromone Research

Sex pheromones have been chemically identified for hundreds of species of insects (for a review see Tamaki, 1985), and the selection of a particular compound is an important choice to be made in adopting electroantennogram (EAG) techniques to monitor atmospheric movements by using pheromone components as tracers. The insect species selected should be large and easy to handle, it should be easy to rear in a colony maintained in the laboratory, and should be hardy and long-lived. American cockroaches (*Periplaneta americana*) are an ideal choice in all of these respects, in that the adults are 10 cm long, can be reared without difficulty, and live for many weeks or months under harsh environmental conditions. The sex pheromone of this insect consists of two complex compounds, periplanone–A and periplanone–B, and is quite expensive (approximately $50,000 per gram). Fortunately, pheromone analogs (mimics) that are much less expensive also elicit EAG responses from American cockroaches, and are available commercially in large quantities. Two excellent candidate compounds for use as tracers are bornyl acetate and camphor, both of which have been used successfully as stimuli in previous EAG studies with American cockroaches (Nishino and Manabe, 1983; Nishino, *et al.*, 1980).

Preliminary experiments in our laboratory have verified that bornyl acetate produces EAG responses in American cockroaches (approximately 1–2 mV, consistent with values reported in the literature). These recordings were made with glass capillaries containing saline solution in contact with silver electrodes coated with silver chloride (Roelofs, 1984).

For extended insect monitoring, it is important to establish that the test insect can survive long periods in the field. We have shown that a live cockroach mounted on a portable probe for EAG recording can live 56 days if a water supply is provided.

1. Pheromone concentration studies

We have accomplished a series of EAG studies with male and female cockroaches (*Periplaneta americana*) tested with different amounts of (−)-bornyl acetate in order to determine suitable concentration relationships. The antennae were tested with discrete amounts of (−)-bornyl acetate on filter paper.

To make an estimate of the actual amount of (−)-bornyl acetate in the puff that passes over the antennae, equations developed by Heath *et al.* (1986) for release rates of semiochemicals from polymeric materials were used. The boiling point of (−)-bornyl acetate is 223°C, theis is between those of decyl acetate (244°C) and octyl acetate (210°C). The half-life of a compound on an absorptive polymeric material is given by

$$(\text{In}) \text{ half life} = 8.48 + 0.0202 \times ECLU$$

where $ECLU$ is the number of equivalent chain length units, and is 1000 for decyl acetate and 800 for octyl acetate. Interpolating by boiling point for (−)-bornyl acetate, the expected $ECLU_{BA}$ for bornyl acetate is

$$ECLU_{BA} = 800 + [(223 - 210)/(244 - 210)] \times (1000 - 800) = 876$$

and the half-life is therefore

$$\text{half life}_{BA} = \exp(-8.48 + 0.0101 \times 876) = 1.44 \text{ days}$$

The puffs for the EAG were about 1 minute apart, and since 5 micrograms are expected to be released in 1.44 days (= 2074 min), about 0.1% of the total is released in a single puff. The results of these experiments are shown in the following figures (8 and 9) of antennal response with respect to the estimated load rates of (−)-bornyl acetate

actually passing over the antenna. These tests indicated that with further refinement, we would be able to determine variations in pheromone tracer concentration and hence estimate the transport-diffusion features of the medium.

2. Quantified load rates

Using a unique pheromone trapping and delivery system and gas-liquid chromatographic analyses, we have now quantified these previous load rates in terms of atmospheric concentrations (nanograms/cm^3). The description of this process is outlined below:

(a) **Cryogenic volatile collection:** A cryogenic collection technique similar to that described by Browne *et al.* (1974) was used, based on a pump in which a glass sample tube closed at one end (12 mm by 35 mm) was immersed in a liquid nitrogen bath. An inert caroborundum boiling chip was placed in a clean sample tube, and a glass pipette containing a square of filter paper (1 cm x 1 cm) loaded with a defined amount of bornyl acetate was connected to the sample tube with Teflon tubing. The filter paper was loaded with bornyl acetate immediately before testing by applying 20 microliters of a solution of bornyl acetate in the solvent dichloromethane. As soon as the dichloromethane had volatilized away (about 5 sec), the filter paper was placed in the pipette. The sample tube was then immersed 20 cm into the liquid nitrogen bath. As air condensed in the sample tube, a vacuum was created that pulled air into the sample tube at 300 ml/min (measured with a bubble flowmeter). After 20 minutes the sample tube was removed from the liquid nitrogen bath, and disconnected from the pipette. The sample tube was immediately placed into a snugly fitting Styrofoam sheath that had been precooled in liquid nitrogen. The condensed air boiled away within 20 minutes, allowing liquid nitrogen (b.p. -196°C) and liquid oxygen (b.p. -183°C) to escape slowly, but leaving bornyl acetate behind. The sample tube was then extracted with two aliquots (2 ml each) of dichloromethane to recover

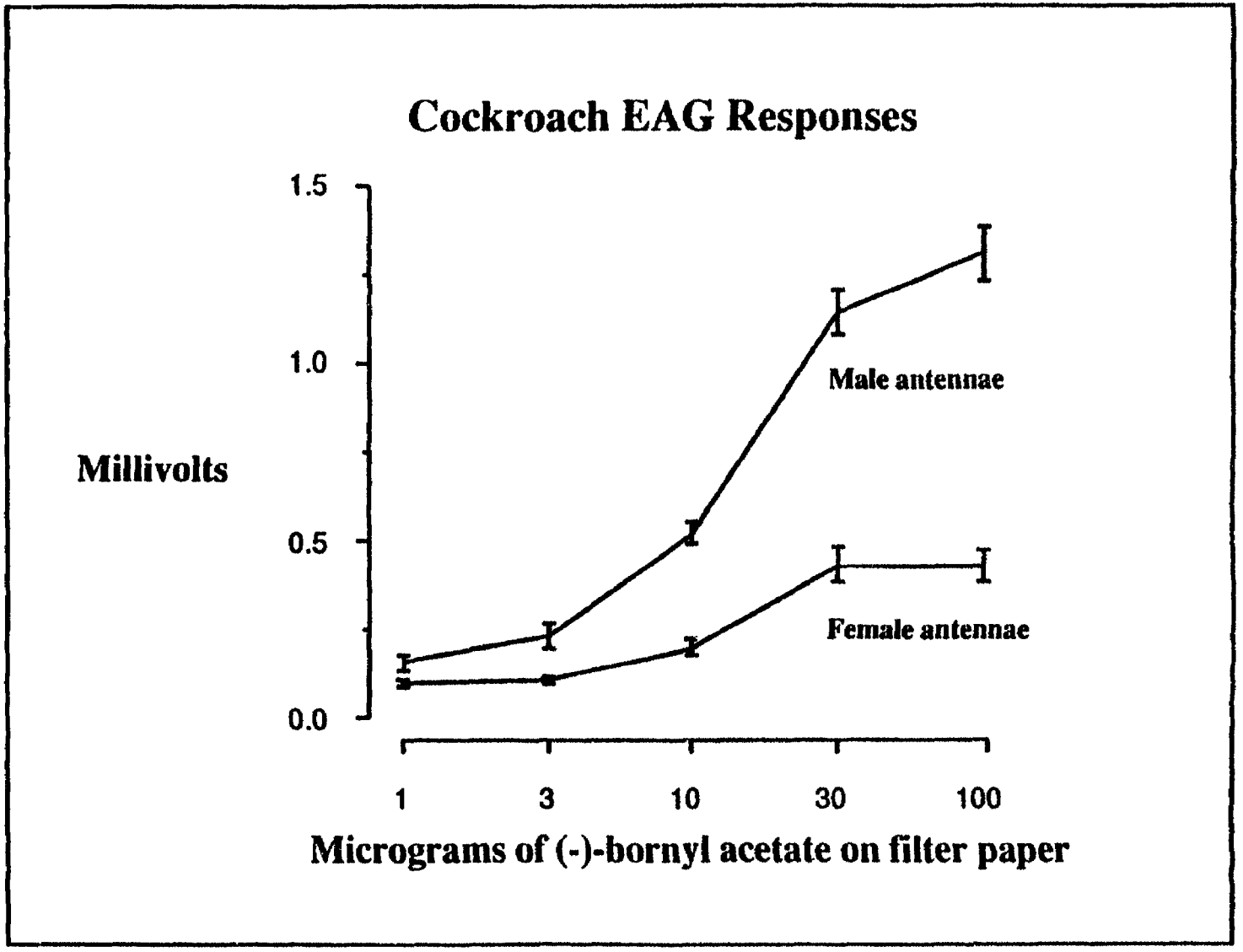


Figure 8: Cockroach EAG Response: Filter paper load.

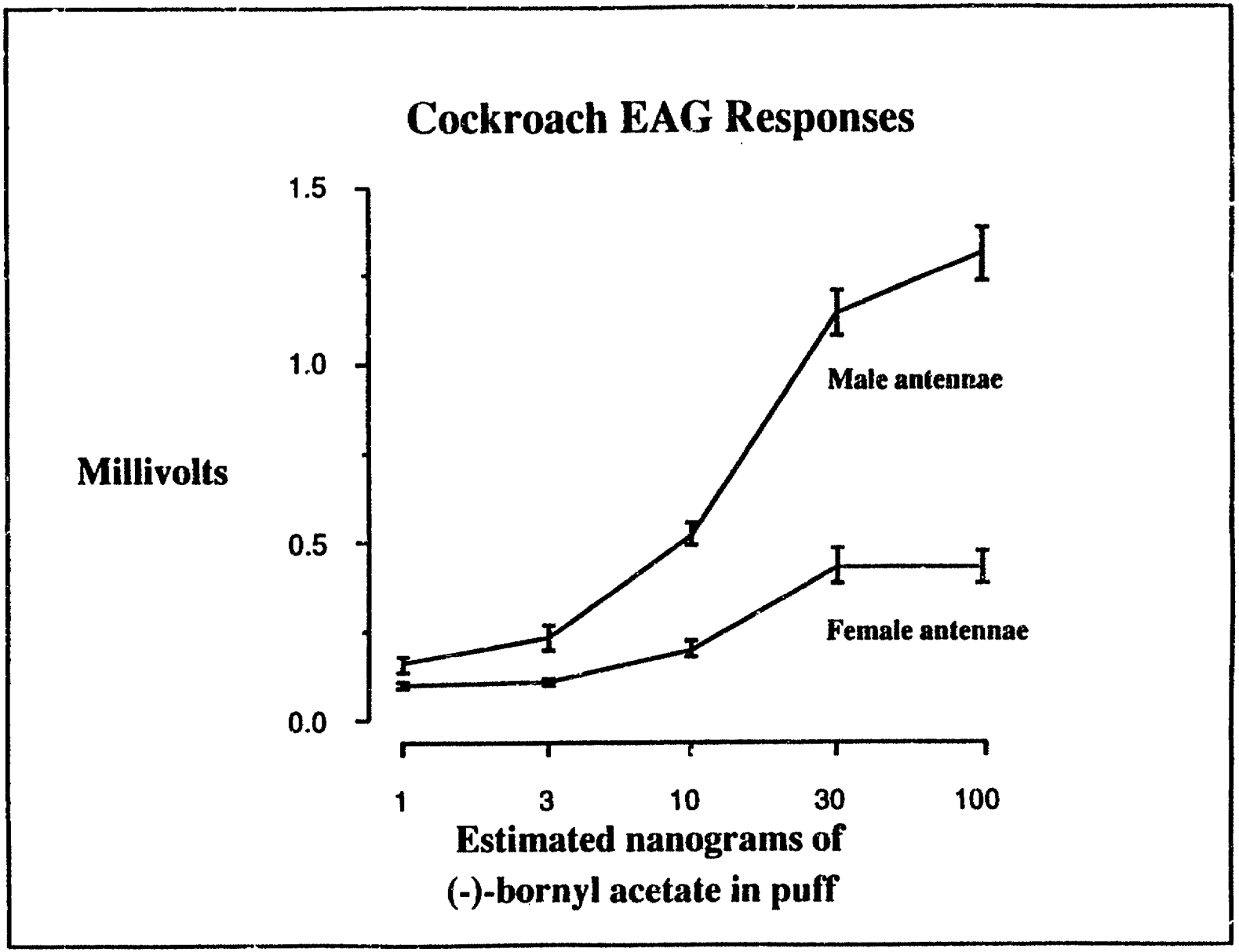


Figure 9: Cockroach EAG Response: Air 'puff' load.

all bornyl acetate that had volatilized into the tube, and an internal standard (methyl palmitate, 1 microgram) was added to allow precise quantitation by gas-liquid chromatography. Gas-liquid chromatographic analyses were conducted with a wide-bore capillary column (methyl silicone, 5 m x 0.5 mm) temperature-programmed from 60° to 240° with a 1 minute delay, with nitrogen as a carrier gas as 20 ml/min, and with flame ionization detection. The retention time of bornyl acetate under these conditions was 5.15 minutes, and the retention time of the internal standard methyl palmitate was 14.68 minutes. Results indicated that all the bornyl acetate could be volatilized and recovered in 10 minutes using this procedure, for five load rates that were tested. These five load rates (1 μg, 3 μg, 10 μg, 30 μg, and 100 μg) were used previously for electroantennogram tests conducted with antennae of the American cockroach, *Periplaneta americana*, as a candidate biosensor.

(b) **Concentration quantification:** The amount of bornyl acetate introduced into an electroantennogram assay by a 1 ml injection of air through a bornyl acetate source was also quantified with the apparatus described above. Instead of connecting the pipette to the sample tube with Teflon tubing, a glass syringe with a silicone rubber fitting was used to inject 1 ml of air through the pipette containing the filter paper impregnated with bornyl acetate, and into the sample tube immersed in liquid nitrogen. The amount of bornyl acetate recovered was determined for each of the above five load rates (Figure 10), to quantify the actual concentrations delivered to the antennae in previous electroantennogram tests. The results are very significant in that we can now not only determine the presence of the pheromone tracer, but we can also estimate the tracer concentration and hence the diffusion directly from the EAG voltage output of the ABS–1.

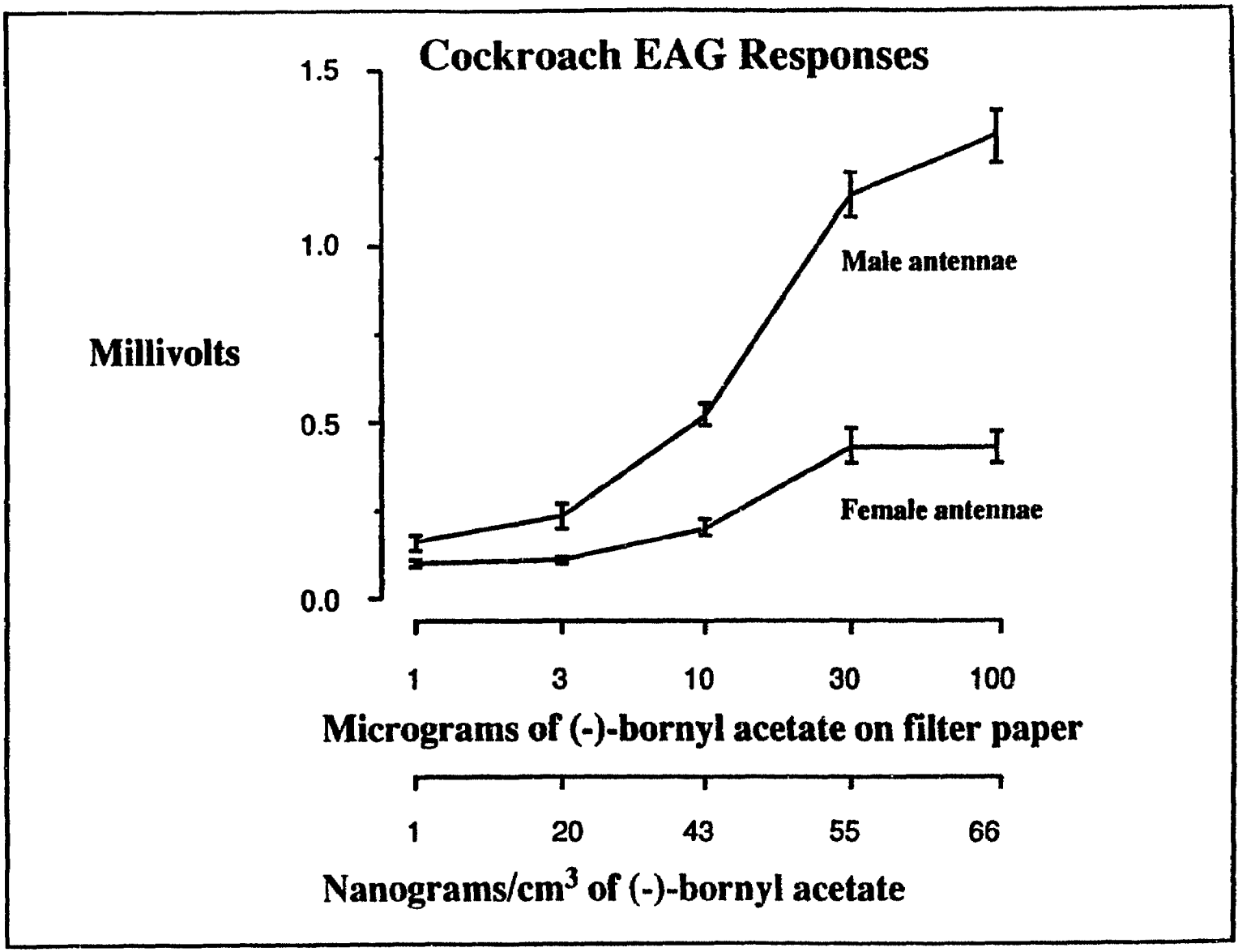


Figure 10: Cockroach EAG Response: Quantified atmospheric load rates.

D. Wind Tunnel Tests of Antenna Sensitivity

In order to simulate an atmospheric field test, several tests of antenna sensitivity were conducted in a low-speed wind tunnel. The tests were designed to study the antenna response to a tracer plume embedded in an atmospheric carrier gas flow.

The insect was placed in a 'body bag' as described above. Electrodes were attached to the antennae, with the platinum wires extending to the edges of the jacket platform. The center wire of a slender coaxial cable (type 174) was attached with a metal clip to the recording electrode, and the coaxial braided sheath was attached with a metal clip to the reference electrode. A slender probe was then made by attaching the jacket platform to the end of a long metal handle (1 × 50 cm) that could be inserted through the side of a liminar-flow tunnel into the center of the tunnel, with the coaxial recording cable extending the length of the probe.

The recording cable was connected to an amplifier (−1000 amplification, $10^{12}\Omega$ input impedance) whose construction was described previously (Bjostad and Roelofs, 1980). The output from the amplifier was displayed with a strip chart recorder (Bausch and Lomb, OmniScribe, Series D5000 recorder). The strip chart recorder was typically run at 1 cm/sec to record the responses of the insect antenna as the probe was inserted into the wind tunnel.

The construction of the wind tunnel was carried out as described by Baker and Linn (1984). A flow of 0.5 m/sec was set in the tunnel, and was characterized by using $TiCl_4$ smoke from a source at the upwind end of the tunnel as a tracer to evaluate tunnel performance. Flow was acceptably laminar as indicated by this technique, and a plume was established that was about 10 cm in diameter at the downwind end of the tunnel. The $TiCl_4$ source was then replaced with a source of the sex pheromone mimic (−)-bornyl acetate, to allow recordings from insect antennae inserted into the tunnel on a probe.

When an insect antennal probe was inserted into the tunnel after a plume of (−)-bornyl acetate had been established, the trace on the strip chart recorder exhibited a sharp deflection of about 1 mV when the antennae were inserted into the plume (Figure 11). The plume consisted of twisted filaments, as indicated by earlier tests with $TiCl_4$ smoke, and

continuous strip chart recording within the plume resulted in rapid deflections and recoveries of the pen, consistent with the filamentous or turbulent nature of the plume. When the probe was removed from the plume, the deflections ceased.

Sample recordings from the wind tunnel are attached. Because the baseline wanders to some extent, due to slowly changing electrolyte concentrations within the insect antennae, we constructed a high-pass filter with a cut-off of 2 Hz to eliminate the wandering baseline. The EAG signal consists almost entirely of higher frequency components, and the high-pass filter effectively improved the quality of the baseline without compromising the information from the EAG signal.

IV. DISCUSSION AND FUTURE RESEARCH

A. Research Results

1. Antennae of the American cockroach *Periplaneta americana* can be used as detectors for air movement, where a pheromone analog for the cockroach (bornyl acetate) is used as a tracer. The American cockroach was selected for initial studies because

   (a) the American cockroach is one of the longest-lived insects

   (b) an inexpensive analog of the sex pheromone (bornyl acetatat) is commercially available.

2. The longevity of antennal detectors made with living cockroaches is very good. With the cockroach antenna interfaced with the detector apparatus, we have obtained useful recordings for at least 5 days. In addition, cockroaches have lived up to 56 days while hooked up to the detector apparatus (although the antennal responsiveness has slowly dropped below the useful range after 5 days), and this indicates that if antennal responsiveness can be improved very long-lived detectors may be achievable. The use of the entire insect, rather than the severed antenna, as has usually been followed previously, greatly extends the cockroach longevity. With the food and water system

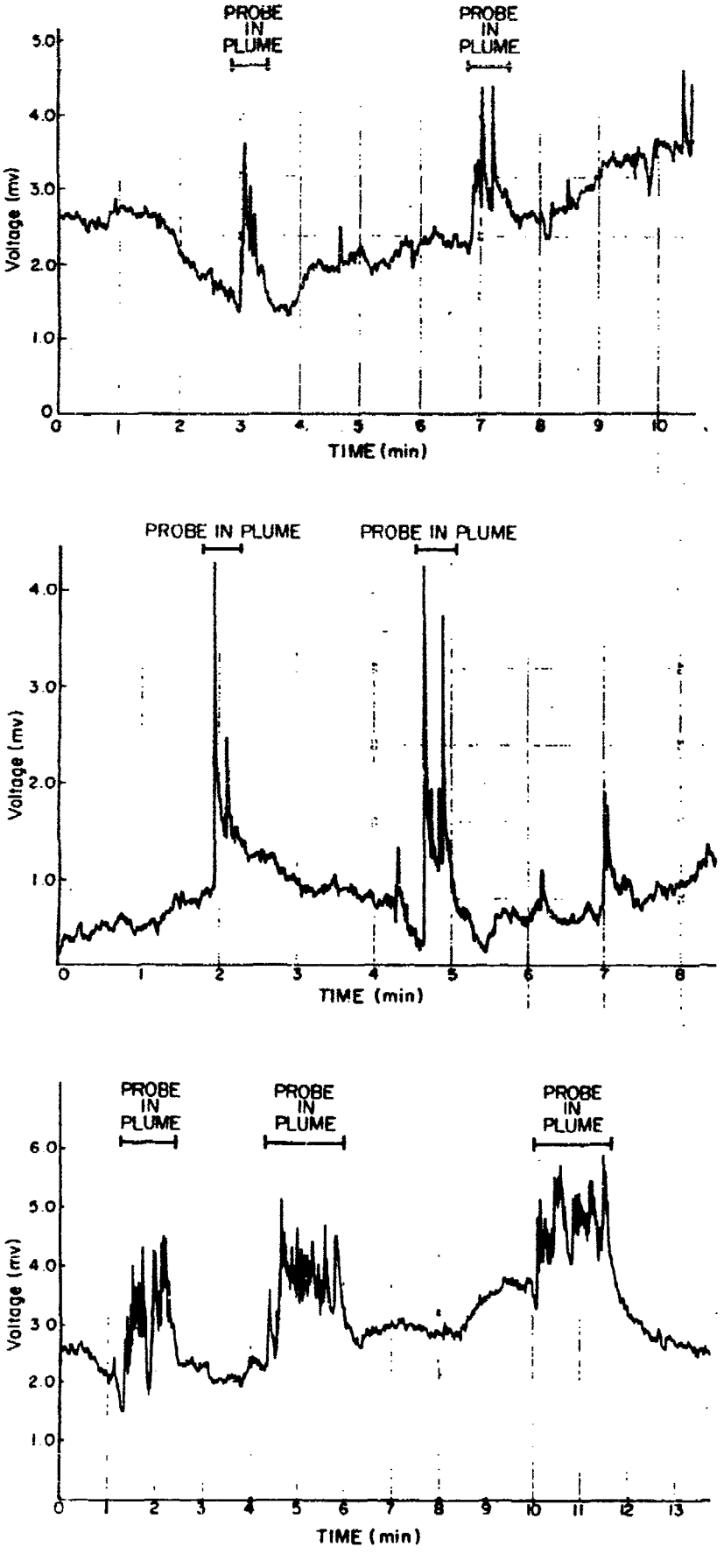


Figure 11: Wind Tunnel Tests of Antenna Sensitivity.

of the ABS–1, we also expect to achieve longer insect lifetimes which will also increase the present continuous 5 day recording lifetime.

3. The main disadvantage of using cockroach antennae as detectors at present is that the antennae have low sensitivity to bornyl acetate, the pheromone analog that was selected as a tracer compound for initial studies. Antennae have only been able to detect bornyl acetate over distances of approximately one meter, as indicated by wind tunnel studies that we have conducted with the cockroach antennal detector. There are two principal opportunities for correction of this problem:

   (a) Use the real sex pheromone of the American cockroach instead of the sex pheromone analog, to extend the range over which the tracer compound can be detected.

      i. Advantages: The antenna of the American cockroach is much more sensitive to the actual sex pheromone (periplanone A) than it is to the pheromone analog that we have tested to date (bornyl acetate). Using 1 ml air puffs over substrates loaded with different amounts of candidate compounds, full response of the antenna requires 100 micrograms of bornyl acetate, but only requires 10^{-3} micrograms of periplanone A (Nishino, 1983). This is a difference of 100,000 times in sensitivity.

      ii. Disadvantages: There is a large difference in the costs of these two compounds, in that the pheromone analog (bornyl acetate) costs 10 cents per gram (Sigma Chemical Company), whereas the actual pheromone component (periplanone A) requires custom synthesis and will cost approximately $10,000 per gram. However, pilot studies would only require about one milligram of periplanone B, and therefore could be conducted relatively inexpensively.

   (b) Use the antenna of the gypsy moth instead of the American cockroach.

      i. Advantages: The antenna of the gypsy moth *Lymatria dispar* is highly responsive to the sex pheromone of this species, disparlure. Using 1 ml air puffs

over substrates loaded with different amounts of disparlure, full response of the antenna requires about 10^{-3} micrograms of disparlure, a sensitivity comparable to the American cockroach (Hansen, 1984). In addition, disparlure is relatively inexpensive, and only costs approximately $70 per gram (Sigma Chemical Company).

ii. Disadvantages: Most adult moths only live a few weeks, and it is likely that the maximum longevity of a detector made with gypsy moth antennae will be approximately 2–3 weeks. For short-term atmospheric studies, this may be adequate. Gypsy moth cultures are somewhat more expensive to maintain than American cockroaches, but they are readily available from a rearing facility at Otis Air Force Base, and it may be possible to have pupae shipped directly for electrophysiological studies.

4. The design development and fabrication of hard contact electrical connections to the insect antenna have produced a robust electronic system suitable for field deployment under various atmospheric conditions. The contact system uses a electrically conducting gel (SCAN) to transfer the electrical activity from the antenna to the silver or platinum wire coils connected to the signal amplification and recording system. This is a new technique which we also believe will be applicable to controlled laboratory conditions.

5. The laboratory experiments indicate that we can now not only determine the presence of the pheromone tracer (wind tunnel/laboratory tests), but we can also estimate the tracer concentration from EAG voltage output of the ABS–1. Thus, atmospheric diffusion estimates are now possible with the pheromone tracer system.

These research results indicate that the original concept of a real-time, pheromone tracer system of the type proposed has in fact been successfully demonstrated. Because of its low-cost and zero background interference feature, the ABS–1 system is indeed unique in the field of atmospheric diffusion detection systems.

B. Future Research

The success of this exploratory research program indicates that a continuous effort in both the pheromone research and the ABS–1 detector system would lead to field test results within a year's time. A small amount of laboratory research is needed to select the proper pheromone or 'mimic' compound of a suitable insect for similar tests as we have accomplished on the cockroach. Completion of the ABS–1 miniature (1000:1) differential amplifiers is needed for a stable, continuous baseline for the remote field measurements. While the cockroach tests indicate a detectability of 1 pp 10^{11} with the actual sex pheromone, improved electronic signal analysis techniques may extend this detectability several orders of magnitude. This would rival or exceed the SF_6 detection capability which is the nearest competitor in real-time tracer detection systems. The pheromone system however experiences a much lower atmospheric background than that for SF_6, i.e. near zero vs 0.5 pp 10^{12}.

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